Determinants of Polyreactivity in a Large Panel of Recombinant Human Antibodies from HIV-1 Infection[1]

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A considerable part of the Ab repertoire is given over to polyreactive Abs capable of interacting with multiple antigenic species. Neither the function of these Abs nor the molecular basis for their activity is known. To address the latter problem, we have compared the amino acid sequences of a large panel ($n = 70$) of polyreactive human monoclonal Fab fragments and conducted a series of engineering experiments on a prototype polyreactive Fab. The Fab fragments were retrieved from combinatorial IgG libraries prepared from the bone marrow of long term asymptomatic HIV-1 seropositive donors. The general features displayed by the panel of IgG polyreactive Abs include 1) skewed V_H family usage with a predominance of V_H1 and V_H4 clones and a paucity of the normally prevalent V_H3 family; 2) use of a variety of different V_H germ-line genes within the context of the family usage and no restriction in D or JH gene usage; 3) skewed V_L gene usage: 75% of Fabs used one of two germ lines; and 4) extensive somatic modification of both heavy and light chains. The importance of the heavy chain, in particular the heavy chain CDR3 (HCDR3), in dictating the polyreactive phenotype was demonstrated for the prototype Fab by chain shuffling and CDR transplantation experiments. In addition, and most strikingly, a constrained peptide based on the HCDR3 sequence was shown to be polyreactive and to inhibit binding of the parent Ab to a panel of Ags. A role for conformational flexibility in polyreactivity was suggested by a marked temperature dependence of Ab recognition of Ag. One Ab was shown to be polyreactive at 37°C, but was apparently monoreactive at 4°C. We hypothesize that Ab polyreactivity is associated with conformationally flexible HCDR3 regions in the context of certain favorable framework configurations. *The Journal of Immunology,* **1996, 157: 739–749.**

A major part of the Ab repertoire in humans consists of Abs reacting with a variety of auto- and exogenous Ags, including proteins, nucleotides, polysaccharides, and lipids (1). Although the existence of these polyreactive Abs has been known for almost a century, they have received limited attention. There is a perception that the basic Ab-Ag interaction involved here is a nonspecific "stickiness" that becomes significant in the context of the polyvalent IgM molecule, since most polyreactive Abs are of the IgM class. However, Ichiyoshi and Casali have shown that a polyreactive IgM can be class switched to IgG with the maintenance of the polyreactive phenotype (2). Furthermore, Casali and co-workers have generated a panel of human polyreactive IgG Abs using EBV transformation of B cells, and we have produced a panel of polyreactive IgG Fabs using phage display technology (3, 4). Therefore, there is an increased appreciation that polyreactivity, characterized by binding affinities in the range from 10^5 to 10^7 M^{-1} for a variety of Ags, can be a property of single Ab-combining sites (5, 6). It is an intriguing problem for structural biology to understand how polyreactivity is achieved at the molecular level.

The weight of the evidence implies that many different Ig variable genes can be used to generate polyreactivity; there is no indication of specific polyreactive Ab encoding variable regions (7). Rather, it appears that many of the variable region genes used in specific Abs to foreign Ags are also used by polyreactive Abs. Most polyreactive Abs are encoded by V genes in near germ-line configuration (8–10). This probably reflects the fact that most polyreactive Abs isolated are IgMs, since the IgG polyreactive Abs studied are generally somatically mutated (3). Recent gene swapping studies have suggested that the heavy chain, in particular the heavy chain CDR3 (HCDR3),[4] is crucial in polyreactive behavior (2, 11).

The function of polyreactive Abs is still unknown. Several hypotheses have been put forward for the roles of polyreactive Abs in vivo, including 1) the primary line of defense against microorganisms or other invading Ags before a specific immune response is generated; it has been suggested that the specific Ab response evolves from the polyreactive response; 2) regulation of the immune system through an idiotypic network; 3) the clearance of cellular debris; and 4) shielding cellularly expressed Ags that resemble foreign Ags to avoid autoimmune attack.

In several diseases an elevated level of polyreactive Abs is observed (5). This includes HIV-1 infection. We have previously shown that high levels of polyreactive Abs rather than specific autoantibodies are the probable cause of the elevated serum Ab titers to a range of autoantigens seen in HIV-1 seropositive donors (4). We characterized the binding specificities of a set of 38 recombinant autoantibodies isolated from combinatorial Ab libraries

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[4] Abbreviations used in this paper: CDR, complementarity determining region; HCDR3, heavy chain CDR3; EGF, epidermal growth factor; FR, framework region; R/S ratio, replacement (R) to silent (S) mutation ratio.

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from a panel of HIV-1 seropositive donors and expressed on the surface of filamentous phage (4). The human IgG mAb Fab fragments were isolated by affinity selection using immobilized autoantigens chosen primarily because of a reported immune response in HIV-1 patients. The autoantigens included MHC class II, DNA, CD14, ganglioside GD2, and epidermal growth factor (EGF) receptor. Evaluation of the cloned Abs revealed that besides binding efficiently to the Ag against which they were selected, they were also capable of binding to a variety of other auto- and exogenous Ags. Typical Fab affinities for Ag were of such an order, ranging from 10^6 to 10^7 M^{-1}, that in vivo interaction may be of importance. Our evaluation thus found no evidence of specific autoantibodies in HIV-1 seropositive donors. Instead, we argued that the cloned autoantibodies were probably reflecting the prevalence of polyreactive Abs in HIV-1 infection, since far fewer such Abs were isolated from an equivalent library prepared from an HIV-1 seronegative donor.

In this study, we seek to gain some understanding as to what features of Ab structure lead to the polyreactive behavior of the Abs described. In the first instance, we have analyzed and compared the variable heavy and light chain regions of an extended panel ($n = 70$) of cloned polyreactive Fabs from HIV-1 donor libraries for correlates of polyspecificity. The number of human polyreactive Abs available allows rather detailed comparisons to be made. Further, the contributions of different parts of the Ab molecule to polyreactivity have been explored by genetic manipulation. Chain-shuffled libraries, involving the recombination of a given heavy or light chain from a prototypic polyreactive clone with the original library of 10^7 complementary chains have been generated. Special emphasis has been placed on the role of the HCDR3 by grafting experiments and by the synthesis of a cyclic peptide derived from the HCDR3 of a polyreactive Ab. Finally, we have analyzed the effect of temperature on expression of the polyreactive phenotype.

The findings indicate that the cloned polyreactive IgG Ab response is restricted in V_H and V_L family gene usage, that the Abs are generally extensively somatic mutated, that the polyreactive phenotype is temperature sensitive, suggesting the importance of protein flexibility to polyreactivity and that the heavy chain, in particular the HCDR3, plays a major role in the polyreactive phenotype of these Abs. In one case, the HCDR3 could be shown to directly convey polyreactivity, either transplanted into another Ab or as the corresponding peptide.

Materials and Methods

Combinatorial libraries from HIV-1 donors

Isolation of the initial 38 polyreactive Fab fragments from combinatorial IgG1κ libraries of approximately 10^7 members generated on the surface of filamentous phage has been described previously (4). Bone marrow from asymptomatic HIV-1 seropositive individuals was used as the RNA source for the library construction (12, 13).

Chain-shuffled libraries

A shuffled library was prepared by cloning the PCR-amplified heavy chain gene of clone LNA3 into the pComb 3 vector containing the original light chain library to give a heavy-light chain library (LNA3HLn) of 3×10^6 members. Another shuffled library containing the original heavy chain library and the light chain of clone LNA3 was also prepared (LNA3LHn). In this case the light chain was first subcloned into pComb3 containing a tetanus toxoid-binding clone. The heavy chain library was then cloned into this construct to give a library of 5×10^6 members. The subcloning step was used to avoid contamination with and over-representation of the original heavy chain. Selection by panning was conducted as described below.

CDR3 grafting

The CDR3 grafting experiment, using overlap PCR, was performed as previously described (14, 15). In brief, the HCDR3 sequence for the polyspecific Fab LNA3 was grafted onto the heavy chain of the tetanus toxoid-specific Fab, p313, replacing the existing HCDR3 (16). Initially, two separate PCR reactions were performed using the primers R3B and HCDR3 LNA3 (GCG-GTG-TAT-TAC-TGT-GCG-AGA-X-TGG-GGC-CAA), and FTX3 and BfR3U, respectively. The two PCR products were mixed and allowed to anneal to each another. Subsequently, this product was amplified with the primers FX3 and R3B to generate full-length template, which was digested with the restriction enzymes *Xho*I and *Apa*I. The purified digested products were ligated into the *Xho*I- and *Apa*I-digested expression vector *Ara*-2P. This vector has been modified from the *Ara*HA vector (17) by removing the decapeptide tag. The successful grafting was verified by sequence analysis. Purified Fab was subsequently prepared, and binding specificity was assayed by ELISA.

Synthesis of a constrained HCDR3 peptide

The design of the constrained peptide, designated PEPLNA3, was achieved by superposition of the HCDR3 and adjacent FR3 residues of Fab LNA3 onto computational three-dimensional Ab models as previously described (18, 19). In a similar fashion, a constrained peptide, designated PEPSI1, with the amino acid sequence AACARKRGRTTVSWGLVYFDYCA was derived from the HCDR3 and adjacent FR3 residues of a specific anti-DNA Fab SI-1 (20). Peptides were synthesized on a 0.015-mmol scale using F-moc chemistry on a Gilson 422 Multiple Peptide Synthesizer (Gilson Inc., Middleton, WI). Biotinylation was conducted following removal of the N-terminal F-moc group using ImmunoPure NHS-LC-Biotin II (Pierce Chemical Co., Rockford, IL). Cyclization was conducted by treating the peptide resin with thallium trifluoroacetate (Aldrich Chemical Co., Milwaukee, WI) in dimethyl formamide.

Selection of antigen-binding clones from shuffled libraries

Panning of the shuffled libraries was conducted as described previously (13). In brief, four microtiter wells were coated either with 0.2 μg/well purified human MHC class II Ag, type DR1 (kindly provided by Dr. L. Teyton), or with the Fc fragment of human IgG (Sigma Chemical Co.) in PBS, pH 7.4, overnight at 4°C. Alternatively, wells were coated with 0.2 μg/ml human placenta DNA (Sigma Chemical Co., St. Louis, MO) in PBS and dried on the plates at 37°C. The wells were washed twice with PBS and blocked by completely filling the wells with 3% (w/v) BSA in PBS for 1 h. The BSA solution was discarded, and 50 μl of the phage library (typically 10^{11} CFUs) were added to each well and incubated for 2 h at 37°C. The soluble phage were then removed, and the well was vigorously washed 10 times with PBS containing 0.05% Tween-20 (PBS-Tween). Bound phage were eluted with 0.1 M HCl (adjusted to pH 2.2 with solid glycine and containing 0.1% BSA) by pipetting up and down several times. Eluted phage were neutralized with 2 M Tris base and used to infect 2 ml of XL1-Blue (Stratagene, La Jolla, CA) for 15 min. Medium containing antibiotics and helper phage were then added sequentially as described above to amplify the diluted phage. The resulting phage library was reapplied to the Ag to initiate a new round of biopanning. Following four rounds of panning, the selected libraries were converted to libraries of phagemids secreting soluble Fabs and screened for binding to the selecting Ag.

ELISA analysis of soluble Fab fragments and peptide

Fabs were prepared as bacterial supernatants through a freeze-thawing procedure, as reported previously (4). To assess initial specificity, supernatants were screened in an ELISA system (13). To examine the specificity of the Fab fragments in more detail, purified Fabs were used as described below. Coating of ELISA wells with different auto- and exogenous Ags at a concentration of 0.1 μg/well and subsequent blocking were conducted as previously described (4). The Fabs were tested against human MHC class II Ag and purified EGF receptor (kindly provided by Dr. A. Komoriya); CD14 (kindly provided by Dr. A. Moriarty); human placenta DNA, tetanus toxoid, and ganglioside GD_2 (Sigma Chemical Co.); the Fc fragment of human IgG, BSA, and OVA (Pierce); and human transferrin (Organon Teknika-Cappel, Durham, NC). Fab was incubated with Ag for 2 h at 37°C followed by washing 10 times with PBS-Tween (GD_2 was washed with PBS-BSA). Bound Fab fragments were detected with an alkaline phosphatase-labeled goat anti-human IgG Fab Ab (1/500 in PBS (Pierce)), incubated for 1 h at 37°C, and visualized with *p*-nitrophenyl phosphate substrate (Sigma Chemical Co.) as monitored at 405 nm. For the temperature-dependent experiment, Fabs and alkaline phosphatase-labeled goat anti-human IgG Fab Ab were incubated at 4 or 37°C. For the peptide studied, visualization of binding of the biotinylated peptide was conducted by the avidin-biotin peroxidase complex detection system (Vector Laboratories, Burlingame, CA).

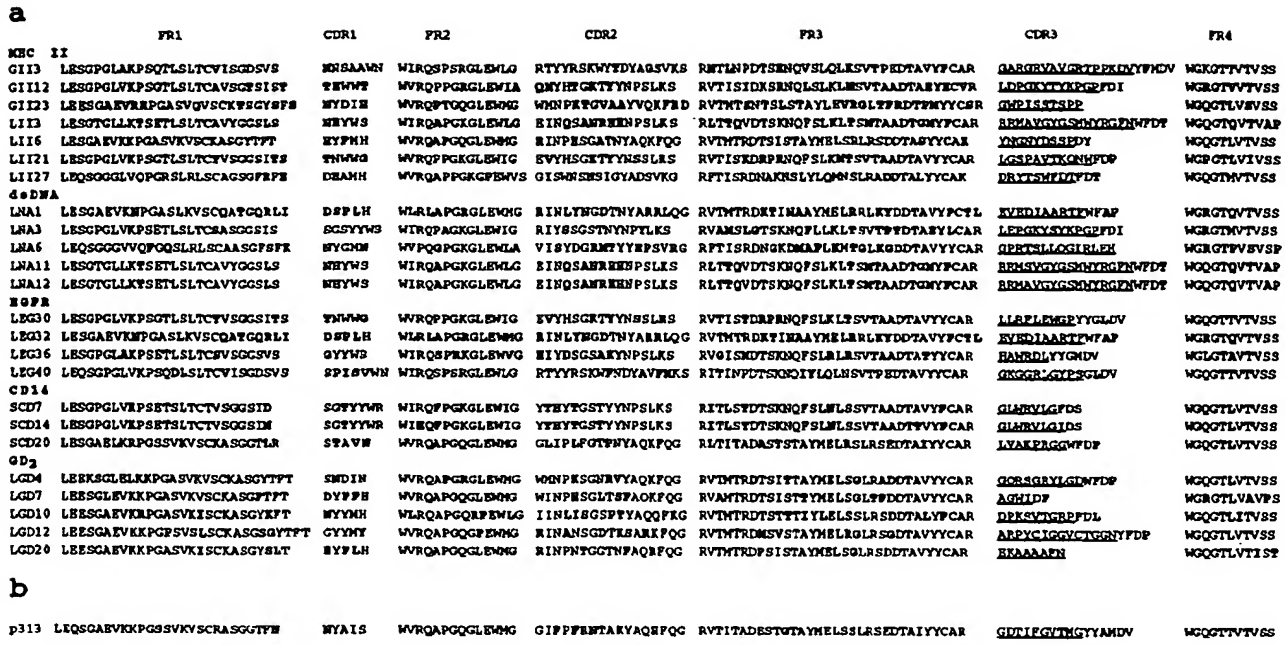

FIGURE 1. Amino acid sequences analysis of V_H domains of the polyreactive Fab fragments isolated from HIV-1 seropositive donor libraries (*a*) and a tetanus toxoid-specific Fab isolated from a healthy donor (*b*). Residues believed to have arisen from somatic mutation (deduced from comparison to the closest germ-line sequence) are in boldface. The D segment and N additions are underlined. The sequence designation of the polyreactive clones is as follows: the first letter indicates the library donor identifier, and the second and third letters indicate the autoantigen against which they were selected (NA, DNA; EG, EGF receptor; II, MHC class II; CD, CD14; GD, ganglioside GD2).

Competition ELISAs

Relative Fab binding affinities were estimated by inhibition ELISAs, using the method of Friguet et al. (21) with modifications. Ags were coated onto ELISA wells and blocked with BSA, as described above. Fab fragments, which had been determined by titration experiments to produce 75% of the maximum binding, were incubated with free competing Ag (10^{-11}–10^{-5} M) or the constrained peptide for 2 h at 37°C. Bound human Fab were detected as described above.

Preparation of purified Fab fragments

Bacterial cultures of the Fabs were purified as previously described (22). In brief, soluble Fab was purified from bacterial supernatants by affinity chromatography using a sheep anti-human Fab fragment matrix (Schleicher & Schuell, Keene, NH). The column was washed with a PBS solution, and Ab was eluted in 0.2 M glycine-HCl buffer, pH 2.2, and immediately brought to neutral pH with 1 M Tris-HCl, pH 9.0. The CDR3-grafted Fab expressed in the *Ara*2P vector was grown in superbroth containing chloramphenicol (30 μg/ml) and $MgCl_2$ (20 mM) at 37°C. Protein expression was induced with 0.25% arabinose and 4 μM cAMP, and grown at 30°C overnight.

Nucleic acid sequencing

Double stranded DNA was used as template in sequencing reactions conducted on a 373A automated DNA sequencer (Applied Biosystems, Foster City, CA) using a Taq fluorescent dideoxynucleotide terminator cycle sequencing kit (Applied Biosystems). Sequencing primers for the heavy chain were SEQGz (5′-GAAGTAGTCCTTGACCAG-3′), hybridizing to the (+) strand, and the T3 primer (5′-ATTAACCCTCACTAAAG-3′), hybridizing to the (−) strand. For the light chain, primer SEQKb (5′-ATA GAAGTTGTTCAGCAGGCA-3′) hybridizing to the (+) strand and primer KEF (5′-GAATTCTAAACTAGCTAGTTCG-3′) were used.

Sequence analysis

Comparison to reported Ig germ-line sequences from GenBank (September 1994) and EMBL (June 1994) was performed using the Genetics Computer Group (GCG) Sequence Analysis program. For analysis of D and J segments, the sequences were compared with folders containing the present reported D, JH, and JK germ-line sequences using the Mac Vector suite of programs (IBI, New Haven, CT). The sequence assigned to the D segments was the nucleotide sequence where the most 5′ nucleotide followed codon 94 of the heavy chain variable region according to Kabat et al. (23) and the most 3′ nucleotide was the last unidentified nucleotide before the sequence matched one of the published germ-line JH genes.

Results

The generation of combinatorial libraries from bone marrow from three long term HIV-1 seropositive donors shown to have high Ab titers to a range of autoantigens has been previously described (4). That report describes the isolation of 38 polyreactive human Fabs by panning the libraries against a range of autoantigens. In this report, the V_H-D-J_H and Vκ-Jκ segments of each of these Fabs were sequenced and analyzed together with a number of Fabs generated in chain-shuffling experiments.

Heavy chain sequence analysis

The analysis of the V_H sequences established that several of the clones obtained by panning against a given Ag had identical sequences, indicative of specific enrichment for these Abs. Twenty-two different heavy chains were isolated. The deduced amino acid sequences are shown in Figure 1*a*. In contrast to our observations from analysis of the anti-gp120 response in one of the long term HIV-1 seropositive donors, we did not find groups of sequence-related clones. Interestingly, in two instances the same Fab was isolated by selection on two different Ags. A heavy chain sequence retrieved from panning the L library against DNA was also retrieved by panning the same library against MHC class II Ag. Another heavy chain sequence obtained from panning against DNA was retrieved by panning against EGF receptor. These findings strongly suggest that the repertoire of polyreactive Abs is restricted. None of the clones showed any resemblance to the virus-specific Abs previously retrieved from these libraries. Detailed analysis of the polyreactive heavy chain sequences is described below together with the sequences resulting from chain shuffling.

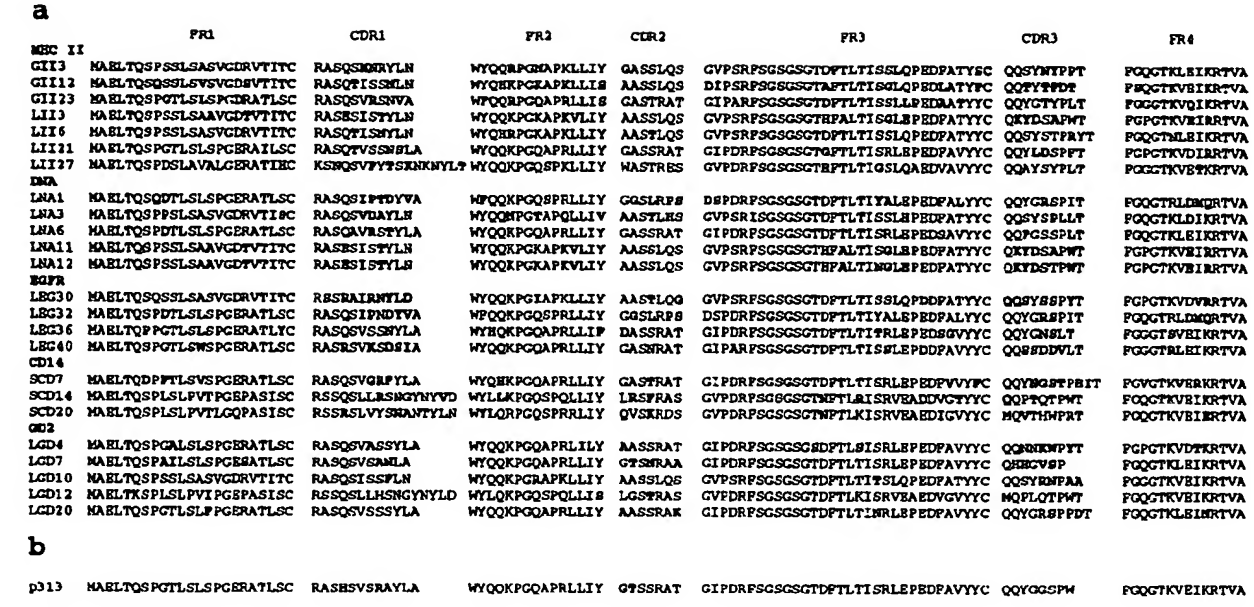

FIGURE 2. Deduced amino acid sequences of the light chain variable domains corresponding to the heavy chains of the polyreactive and specific Fabs depicted in Figure 1. Residues believed to have arisen from somatic mutation (deduced from comparison to the closest germ-line sequence) are in bold.

Light chain sequence analysis

The light chain amino acid sequences, corresponding to the heavy chain sequences depicted in Figure 1*a* are shown in Figure 2*a*. Analysis of the light chain sequences revealed that all the sequences were distinct, except in two instances. Those two light chains were paired with two identical heavy chains, but were retrieved by selection using different Ags: DNA and MHC class II, and DNA and EGF receptor, respectively. Detailed analysis of light chain sequences is conducted below.

Shuffling of the heavy and light chain of a single clone against a library of complementary chains

One of the polyreactive Fabs from the panel, LNA3, was chosen for structural evaluation. This Fab has been shown to exhibit marked polyreactivity in interacting with several Ags with apparent affinity of the order of 10^6 M^{-1} (4, 20) as illustrated in Figure 3*a*. The heavy and light chain contributions to the polyreactive phenotype of this Ab were probed by chain-shuffling experiments. A shuffled library (LNA3HLn) was prepared by cloning the PCR-amplified heavy chain gene of Fab LNA3 into the pComb3 vector containing the original light chain library of 10^7 members. Similarly, another shuffled library (LNA3LHn) was constructed by cloning the PCR-amplified light chain gene of clone LNA3 into the pComb3 vector containing the original heavy chain library of 10^7 members. The heavy and light chain-shuffled libraries were then panned for four rounds against DNA, MHC class II, and the Fc part of IgG, and a panel of Fabs was retrieved that bound to the selecting Ag by ELISA. These Fabs also bound to the set of auto- and exogenous Ags used previously and, therefore, were clearly polyreactive. The heavy chains from 20 polyreactive Fabs selected from the LNA3LHn library were sequenced to reveal that several of the clones were identical. In some cases, the same heavy chain was found in clones selected by panning against a different Ag. Further, it was found that all the Fabs exhibiting the highest OD values in the binding assay and more than half of the total isolated Fabs had heavy chain sequences identical with the heavy chains isolated in the original panning experiment. These included the heavy chain sequences of Fabs LNA12, LGD4, and the original parent Fab, LNA3. Binding specificities of Fab LNA3L/LNA12H containing the light chain of LNA3 and the heavy chain of LNA12 are shown in Figure 3*b* together with the comparable specificities for the two parent Fabs LNA3 and LNA12 (Fig. 3, *a* and *c*). The selection of the original heavy chains from the shuffled library is a further indication that these particular heavy chains contain special features important for polyspecific binding. The deduced amino acid sequences of the seven new heavy chains are depicted in Figure 4*a*. Eighteen polyreactive Fabs were also isolated from the LNA3HLn library. Sequence analysis of the light chains of these Fabs demonstrated that all the light chains were different from each other and also different from the original panel of 24 light chain sequences. The deduced amino acid sequences of these additional light chains are shown in Figure 4*b*.

Grafting of polyspecific HCDR3

We further evaluated the importance of the CDR3 of the heavy chain for the polyreactive phenotype. This was performed by grafting the DNA encoding for the HCDR3 of the polyspecific Fab LNA3 into the expression vector *Ara*-2P containing the DNA encoding the monospecific IgG tetanus toxoid-binding Fab p313, thus replacing the existing HCDR3. As shown in Figure 3*d*, Fab p313 binds with high affinity and specificity to tetanus toxoid. Fab p313 was selected because its V_H and V_L family usages are different from those of Fab LNA3. However, the two Fabs have identical amino acid sequences just adjacent to the HCDR3 (positions 92–94 and 103–104). These flanking sequences may be of importance for the structure of the third hypervariable loop (24). The deduced amino acid sequences of p313 are depicted in Figures 1*b* and 2*b*. The heavy chain of p313 shows 93.3% homology with the germ-line gene DP-10 belonging to the V_H1 family and the light chain has κv325 (VκIII) as closest V_L germ line. Fab LNA3 uses a V_H4 heavy chain germ line (hcak) and the light chain vk02/012 (VκI as th closest germ line; Tables I*a* and II*a*). The binding specificity of the LNA3 HCDR3-grafted Fab (LNA3/p313) was tested in ELISA against a panel of exogenous and autoantigens, as shown

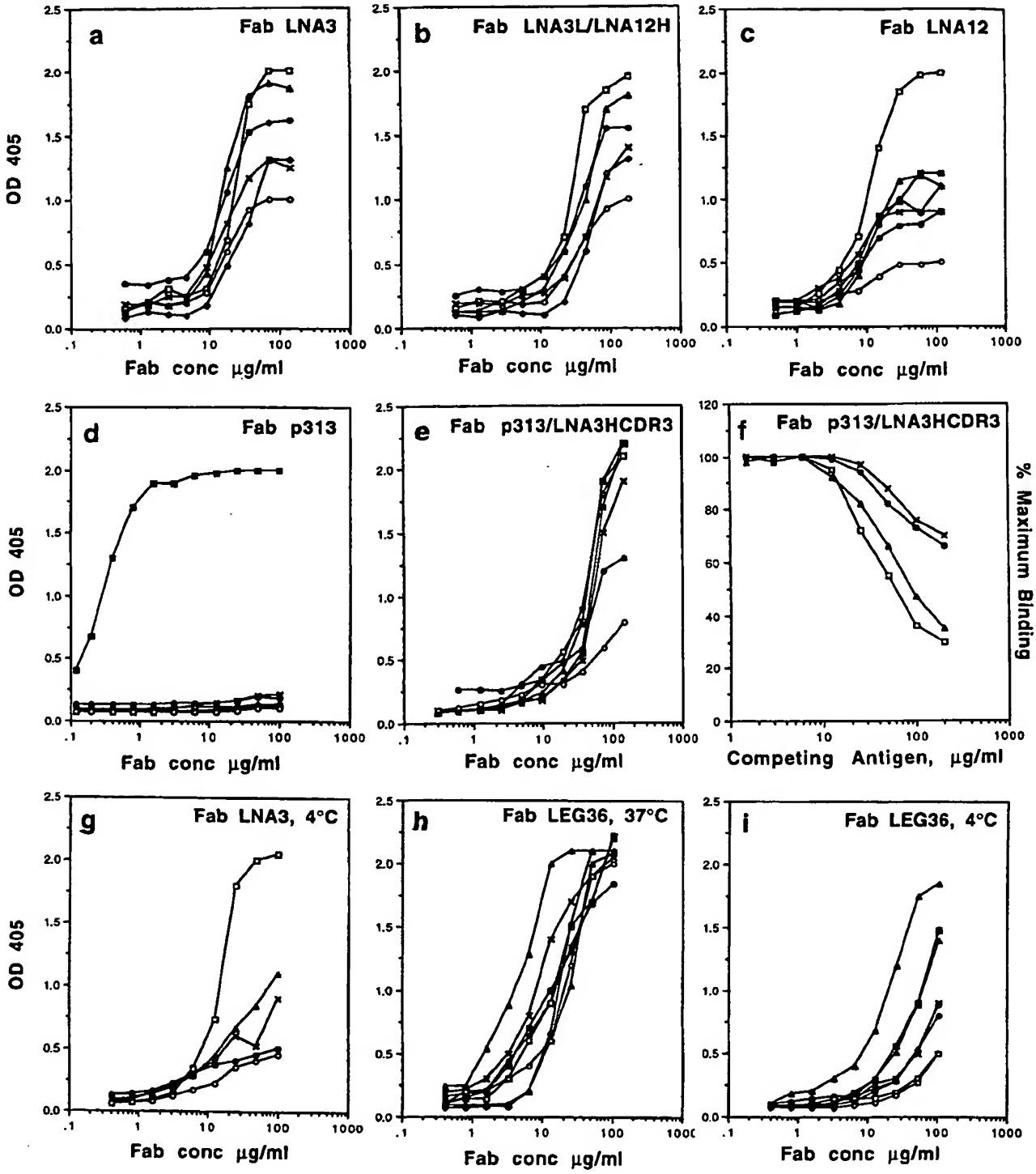

FIGURE 3. Binding specificity of a panel of polyspecific human Fab fragments. The Fabs were assayed for binding to solid phase human DNA (□), OVA (▲), human transferrin (●), BSA (○), tetanus toxoid (■), EGF receptor (△), Fc fragment of human IgG (×), and ganglioside GT_1 (◇). Fab LNA3 (*a*) and Fab LNA12 (*c*) were both selected from an HIV-1 seropositive donor library (library L) by biopanning against human placental DNA. The LNA3L/LNA12H Fab fragment (*b*; containing the light chain of Fab LNA3 and the heavy chain of Fab LNA12) was retrieved from the LNA3LHn shuffled library by panning against MHC class II. Fab p313 (*d*) was selected from a healthy donor library using tetanus toxoid as the selection Ag. The chimeric Fab fragment LNA3/p313 (*e*) was generated by grafting the HCDR3 of the polyreactive Fab LNA3 into the Fab p313, replacing the existing HCDR3. *f*, Inhibition of the binding of the chimeric Fab fragment LNA3/p313 to solid phase human DNA by soluble homologous (human DNA; □) and heterologous (human transferrin (●), OVA (▲), and the Fc fragment of human IgG (×)) Ags. Fab LNA3 binding (*g*) measured at 4°C compared with that measured at 37°C (a) is shown. Fab LEG36 binding was measured at 37°C (*h*) and 4°C (*i*).

FIGURE 4. *a*, Deduced amino acid sequences of the heavy chain variable domains of the polyreactive Fabs isolated from the shuffled library LNA3LHn. *b*, Deduced amino acid sequences of the light chain variable domains of the polyreactive Fabs isolated from the shuffled library LNA3HLn. Residues believed to have arisen from somatic mutation (deduced from comparison to the closest germ-line sequence) are in bold. HCDR3 residues probably arising from D segment(s) and N additions are underlined.

Table I. *Comparison of the heavy chain variable regions of polyreactive Fab fragments to closest germline V_H, D and J_H genes*[a]

Clone	V_H Family	Germline Donor	% Nucleotide Homology	R/S Mutations FR	R/S Mutations CDR	D Segment	J_H
A							
GII3	VI	V6-01/6-1G1	93.5	2.0 (12)	>3.0 (3)	DN1	J_H6d
GII12	IV	DP-70	86.7	1.0 (20)	4.5 (11)	DLR1	J_H3b
GII23	I	DP-15	80.7	2.6 (25)	2.5 (14)	D5′	J_H4b
LII3	IV	V_H4.21	84.6	1.4 (22)	2.0 (12)	D21/10	J_H5a
LII6	I	DP-75	95.2	0.5 (6)	>4.0 (4)	DM1	J_H4b
LII21	IV	DP-70	90.0	1.3 (18)	3.5 (9)	DN1	J_H5a
LII27	III	DP-31	95.2	4.0 (5)	0.7 (5)	D4	J_H3b
LNA1	I	DP-75	78.7	2.2 (29)	2.8 (15)	DN4	J_H5a
LNA3	IV	3d279d	93.8	5.5 (13)	>2.0 (2)	DK4	J_H3b
LNA6	III	3019b9	79.6	1.8 (26)	1.8 (14)	DXP′1	J_H4b
LNA11	IV	V_H4.21	84.6	1.4 (22)	2.0 (12)	DN1	J_H5a
LNA12	IV	V_H4.21	84.6	1.4 (22)	2.0 (12)	DN1	J_H5a
LEG30	IV	V_H4.19	89.6	0.7 (18)	3.5 (9)	DXP4	J_H6b
LEG32	I	DP-75	78.7	2.2 (29)	2.8 (15)	D4	J_H5a
LEG36	IV	V71-4	89.1	1.5 (15)	2.5 (7)	DXP′1	J_H6b
LEG40	VI	V6-01/6-1G1	92.1	0.7 (10)	2.3 (10)	DK1	J_H6b
SCD7	IV	V_H4.33	89.5	1.3 (14)	2.5 (7)	D4	J_H5a
SCD14	IV	V_H4.33	88.6	2.0 (18)	2.5 (7)	D4	J_H5a
SCD20	I	DP-10	90.7	0.8 (11)	3.0 (8)	D6	J_H5a
LGD4	I	DP-15	94.1	2.0 (9)	4.0 (5)	D4	J_H5a
LGD7	I	DP-75	89.6	0.8 (14)	3.5 (9)	D5′	J_H4b
LGD10	I	21-2	87.0	2.2 (19)	2.0 (12)	DA1	J_H4b
LGD12	I	DP-75	88.8	2.8 (15)	5.0 (6)	D1	J_H4b
LGD20	I	DP-75	92.5	1.7 (8)	7.0 (8)	D2	J_H4b
				1.8	>3.0		
B							
LSC2	III	VH26/v3-23	90.0	1.3 (16)	5.0 (6)	D1	J_H4b
LSC3	V	VH251	91.1	2.0 (12)	6.0 (7)	D21/10	J_H4b
LSC6	I	DP-15	88.1	1.3 (18)	0.8 (9)	D2	J_H5a
LSC9	IV	V_H4.11	93.1	2.0 (9)	7.0 (8)	DN4	J_H6b
LSC10	III	9-1	92.9	4.0 (10)	3.0 (8)	DK1	J_H4b
LSC11	IV	3d279d	92.8	2.0 (15)	>3.0 (3)	D2	J_H4b
LSC12	IV	V_H4.35	88.2	3.7 (14)	>11.0 (11)	D2	J_H4b
				2.4	>5.2		

[a] A. Clones isolated during initial selection. B. Clones isolated from the LNA3LHn shuffled library. Figures within parentheses indicate the total number of mutations in each instance.

in Figure 3*e*. Although the chimeric Fab bound less well to the panel of Ags than the original LNA3 Fab (Fig. 3*a*), significant binding was observed. The monospecific binding pattern of the p313 was changed to a polyreactive binding pattern by grafting only the CDR3 of LNA3 into the Fab. To verify the binding data and measure the apparent affinity of the chimeric Fab LNA3/p313,

FIGURE 5. Schematic representation of the Fab LNA3 HCDR3-derived peptide PEPLNA3. The sequence between the cysteines corresponds to the HCDR3 sequence of Fab LNA3, with the inclusion of an arginine at the N-proximal end that may form a salt bridge to the C-proximal aspartate.

competition experiments with soluble Ag were performed. As shown in Figure 3*e*, the apparent affinity of Fab LNA3/p313 for different Ags was on the order of 10^5 to 10^6 M^{-1}.

Analysis of a constrained peptide derived from the HCDR3 of Fab LNA3

The previous experiments strongly indicated that the HCDR3 of Fab LNA3 was of major importance for the polyreactive phenotype. To evaluate whether the HCDR3 in itself was able to mimic the binding pattern of Fab LNA3, a constrained peptide (PEPLNA3) corresponding to the HCDR3 of LNA3 was synthesized. The HCDR3 and adjacent framework regions were superimposed as far as possible on HCDR3 of published Ab structures for positioning of the cysteines used for the cyclization using computer graphics display. As shown in Figure 5, the 21-mer peptide, PEPLNA3, including the entire HCDR3, an N-proximal arginine from the HFR3 region, which has been found by others to be important for the correct folding of HCDR3 peptides (25); two cysteines for cyclization of the peptide; and an alanine tail to permit biotinylation of the peptide. The binding specificity of PEPLNA3, as tested in an ELISA against a panel of Ags, demonstrated the ability of the peptide to bind multiple Ags (Fig. 6*a*). Further, the ability of the peptide to inhibit the binding of Fab LNA3 and Fab LNA3/p313 to a panel of Ags was studied. As shown in Figure 6*b*, increasing concentrations of PEPLNA3 were able to inhibit the binding of Fab LNA3 with an apparent affinity in the micromolar range. Similarly, PEPLNA3 was also able to inhibit the binding of Fab LNA3/p313 (data not shown). A biotin-conjugated constrained peptide (PEPSI1) was derived from the HCDR3 of an anti-DNA-specific Fab fragment (20) as a control. This peptide exhibited no significant binding to a panel of Ags, including human DNA, OVA, HIV-1 gp120, ganglioside GT1, Fc fragment of IgG, and BSA (data not shown).

The influence of temperature on the binding properties of polyreactive Fab fragments

The effect of temperature on polyreactive behavior was evaluated by examining the binding of two Fab fragments, Fab LNA3 and Fab LEG36, to a panel of Ags at 4 and 37°C. As shown in Figure 3, *a* and *h*, both Fab fragments exhibited the polyreactive phenotype at 37°C, although Fab LEG36 had a preference for the EGF receptor. At 4°C, this preference was more pronounced, although the affinity of Fab LEG36 for all Ags was lower at this temperature (Fig. 3*i*). At 4°C, Fab LNA3 appeared to be nearly monoreactive (Fig. 3*g*), showing a strong preference for the Ag used for the original selection, i.e., human DNA.

Heavy chain gene analysis

Comparison of the V_H genes of the initial 24 polyreactive Fabs to reported Ig germ-line V_H sequences from GenBank and EMBL revealed a striking deficiency of Fabs using the V_H3 family, which is the largest of all the V_H families (Table I*a*). Only 2 of 24 clones used this family. Instead, the clones were of the V_H1 and V_H4 families; both were represented by 10 clones. The primers we have used typically lead to roughly equal representation of V_H1 and V_H3 families in unselected libraries. Further, we found no deficiency of V_H3 family usage in the anti-viral Abs retrieved from the same libraries, including Abs to different epitopes on HIV-1 gp120. None of the Fabs used the smaller V_H families 2 and 5, whereas two clones were of the V_H6 family. This latter family has been described to be associated with polyreactive autoantibodies in both regular autoimmune disease and healthy donors (26). Analysis of the V_H family usage of the 20 polyreactive Fab obtained from the light chain-shuffled library revealed that only two Fabs used heavy chains from the V_H3 family (Table I*b*). Of the other new heavy chain sequences, three used V_H4, one used V_H1, and one used the smaller V_H5 family. Further analysis of V_H gene usage of the polyreactive Fabs revealed no particular restriction in germ-line usage within the context of the V_H families employed. Sixteen different germ-line genes were represented. The most frequently used germ line was DP-75, used by six Fabs. Three Fabs had DP-70 as the closest germ line.

The V_H gene analysis also demonstrated that the clones were extensively somatically mutated or used germ-line genes not yet described. The homologies of the V_H of the polyreactive Fabs to the closest known germ-line genes are range from 78.7 to 95.2% (average, 89.0%). Somatic mutations in the FRs and CDRs were analyzed by measuring the replacement (R) to silent (S) mutation ratio (R/S ratio) for the CDR (CDR1 and 2) and FR (FR1, -2, and -3), which were >2.9 and 1.9, respectively. These values are similar to those reported for a number of other human Abs (27–29).

To probe for general trends in somatic mutation in V_H genes of the polyreactive Abs, we first analyzed the amino acid replacement frequency (mutated amino acids/total amino acids) for the individual CDR and FR regions using published germ-line sequences. The amino acid replacement frequencies of the different regions were: FR1, 11.4%; CDR1, 36.9%; FR2, 8.9%; CDR2, 27.4%; and FR3, 20.3%. Overall, these replacements are similar to those reported for other human Abs (27–29). The mutated residues are shown in bold in Figures 1 and 4. Additionally, we analyzed the type of amino acids being replaced by somatic mutation. In the CDR1, there was a strong tendency to mutate away from serines, tyrosines, and glycines and replace those with asparagines, threonines, and histidines. In the CDR2, the changes were more unevenly distributed. However, there was a strong tendency to introduce arginine to this CDR, which is not present in most published germ-line configurations. In contrast, serines and asparagines were lost. In the framework regions FR1 and FR2, no clear pattern was established. In FR3, there was a strong tendency to lose serines and valines and acquire threonines, lysines, and arginines.

Since the HCDR3 of LNA3 in the grafting experiment was shown to convey the polyreactive phenotype, we focused our attention on the HCDR3 and closest germ-line D segments of the polyreactive Fabs (Table I). The identification of the closest germ-line D segment proved difficult due to significant somatic modification or use of germ-line D segments not yet described. With this

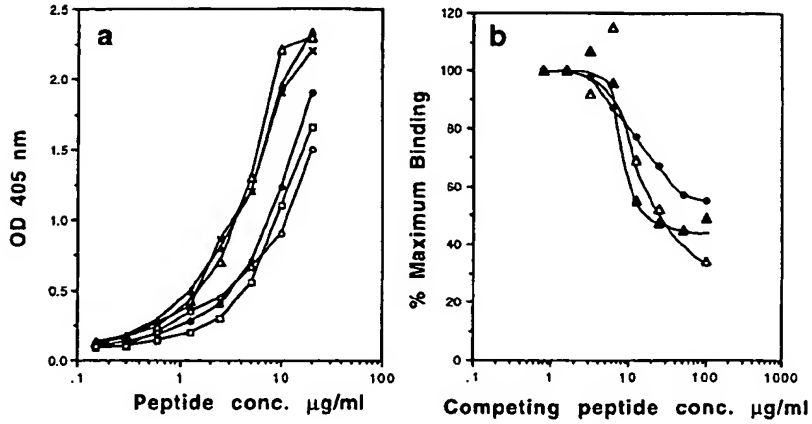

FIGURE 6. *a*, Binding of the Fab LNA3 HCDR3-derived cyclic peptide PEPLNA3 to a panel of Ags (the symbols used are explained in Fig. 3). *b*, Inhibition of the binding of Fab LNA3 (incubated at a constant concentration that in a previous titration experiment yielded 75% maximal binding) to a panel of Ags by increasing concentrations of peptide PEPLNA3.

caveat, a wide panel of different D segments appeared to be used, and no obvious restriction was found. The HCDR3s were further examined for length and amino acid composition. We compared the HCDR3s of 31 polyreactive Fabs with HCDR3s from 32 independent monoreactive Fab clones derived from libraries. These Fabs (eight anti-HSV (30), eight anti-HIV gp120 (31), a second set of eight anti-HIV-1 gp120 (22), and eight anti-DNA (20)) were retrieved from phage libraries generated from HIV-1 donors and from a systemic lupus erythematosus patient. The lengths of the HCDR3s of the polyreactive Fabs varied from 6 to 21 amino acids (average, 13.2), comparable to the HCDR3s from monoreactive Fabs, which are 10 to 20 amino acids in length (average, 13.8). We also scanned the HCDR3s for features that might be expected to contribute to polyreactive behavior, such as clusters of glycines, serines, or prolines, that might permit multiple alternative loop conformations. Although such clusters could be found in two-thirds of the sequences, the polyreactive Abs did not differ greatly in this respect from the monoreactive Abs.

The JH gene usage of the 31 different polyreactive Fabs was also examined (Table II). The most frequently found JH segments were JH4b (40.6%) and JH5a (31.2%). Other JH segments used were JH3b (12.5%), JH6b (9.4%), and JH6d (6.3%). This distribution corresponds closely to the normal distribution of JH segments (32).

Light chain gene analysis

The nucleotide sequences of the light chains of the original 24 polyreactive Fab fragments and the 18 Fabs obtained from the heavy chain-shuffled library were also compared with Vk germline sequences in the GenBank database (Table II). The comparison according to Vk family usage revealed that 26 Fabs used Vk3, 13 used Vk1, two used Vk2, and one used Vk4. Comparison of the sequences to the closest Vk germ-line donor showed that nine different germ-line genes were used. However, two germ lines were strikingly over represented. Thirty-one of the 42 Fabs (74%) used either vk02/012 or kv325. Ten of the 13 Vk1 had vk02/012 as their closest germ-line gene, and 21 of the 26 Vk3 light chains had kv325 as the closest germ-line gene. These figures can be compared with the Vk germ-line usage of 14 anti-gp120 Fabs generated from HIV-1 library L, from which most of the polyreactive Fab fragments were isolated (22). Four clones used the vk02/012, and none used the kv325 germ lines. For 33 anti-gp120 Abs from another HIV-1 library, 7 used the vk02/012, and 13 used the kv325 (31). Therefore, there is some indication that polyreactive Abs show greater preference for vk02/012 and kv325 germ-line genes than specific Abs.

As for the V_H sequences, the V_L sequences were somatically mutated, although slightly less so than the V_H sequences. As shown in Table II, the nucleotide homology to closest germ line ranged from 86.4 to 100%, with an average of 93.4%. For the originally selected polyreactive Fabs, the R/S ratios was >3.5 for the CDR region (DR1 and -2) and 1.7 for the FR region (FR1, -2, and -3). For the Fabs selected from the shuffled library LNA3HLn, the R/S ratios for the CDR region were >3.1 and >1.4 for the FR region. To determine the somatic mutations were located at particular positions, the mutated amino acids were highlighted in Figures 2 and 4. No particular pattern was observed. As shown in Table II, analysis of the Jκ usage revealed no restriction. Nine Fabs used Jκ1, 12 used Jκ2, six used Jκ3, 10 used Jκ4, and five used Jκ5.

Competition of polyreactive Fabs with serum Ab

To provide an assessment of the relative importance of polyreactive Abs in serum responses, a pool of polyreactive Fabs was competed with serum from two HIV-1 seropositive library donors for binding to two Ags, DNA and OVA. Both library donors had high serum Ab titers to a range of autoantigens (donors L and C) (4). Bound serum IgG was detected using a labeled anti-Fc Ab. A pool of three polyreactive Fabs (LEG36, LNA3, and SCD20) at a total Fab concentration of 100 μg/ml inhibited serum IgG binding (serum at 1/100 dilution) by 30 to 60%, indicating the importance of the polyreactive component of the response represented by these Fabs.

Discussion

In this study we have evaluated the gene usage and structural features of a large panel of polyreactive IgG human monoclonal Fab fragments isolated from HIV-1 seropositive donors. The study includes two strengths in this regard. First, the size of the panel (70 Abs) is large, so that general trends can be most readily discerned. Second, the investigation of Fabs means that the effects observed relate to single Ab-combining sites, and multivalency is not a complicating factor.

In comparing gene usage in the panel of polyreactive human Abs, a diverse set of variable genes was found, in agreement with earlier studies on a smaller sample size (reviewed in Ref. 5). The most notable restriction was a paucity of V_H3 genes. No such paucity was observed in Abs to viral pathogens retrieved from the same libraries. It is not possible from these studies to determine whether this restriction applies to all polyreactive Abs or is peculiar to HIV-1 infection. V_H3 depletion has been reported previously in patients with AIDS (33), but was not found in long term HIV-1 seropositive asymptomatic donors (34), who should be more comparable to the library donors. The repertoire of the polyreactive light chains demonstrated a significant over-representation

Table II. *Comparison of the light chain variable regions of polyreactive Fab fragments to closest germline V_L and J_L genes*[a]

Clone	V_L Family	Germline Donor	% Nucleotide Homology	R/S Mutations FR	R/S Mutations CDR	$J\kappa_L$
A						
GII3	I	Vk02/012	95.8	1.5 (5)	>4.0 (4)	2
GII12	I	Vk02/012	91.2	8.0 (8)	0.7 (5)	1
GII23	III	kv328h5	90.5	2.8 (15)	2.0 (3)	4
LII3	I	Vk02/012	91.4	2.0 (12)	0.7 (5)	3
LII6	I	Vk02/012	95.4	0.3 (5)	>3.0 (3)	2
LII21	III	Kv325	94.7	0.3 (10)	>3.0 (3)	3
LII27	IV	Hsigk18	93.5	1.7 (8)	2.5 (7)	4
LNA1	III	Kv325	88.5	3.5 (9)	9.0 (10)	5
LNA3	I	Vk02/012	88.8	1.6 (13)	1.7 (8)	2
LNA6	III	Kv325	96.7	1.0 (4)	>3.0 (3)	2
LNA11	I	Vk02/012	95.8	1.5 (5)	>4.0 (4)	3
LNA12	I	Vk02/012	95.4	2.0 (6)	>4.0 (4)	3
LEG30	I	L11	86.4	0.1 (17)	8.0 (9)	3
LEG32	III	Kv325	88.8	3.5 (9)	9.0 (10)	5
LEG36	III	Kv325	92.6	1.0 (12)	1.0 (4)	4
LEG40	III	Kv325	93.1	0.8 (9)	>6.0 (6)	4
SCD7	III	Kv325	93.3	2.0 (9)	4.0 (5)	2
SCD14	II	A19	92.8	1.0 (10)	4.0 (5)	1
SCD20	III	A17	94.1	1.5 (5)	2.5 (7)	1
LGD4	III	Kv325	93.7	0.6 (13)	>2.0 (2)	3
LGD7	III	Kv325	89.3	0.5 (16)	5.0 (6)	2
LGD10	I	Vk02/012	97.5	1.0 (4)	1.0 (2)	4
LGD12	II	A19	98.0	2.0 (3)	1.0 (2)	1
LGD20	III	Kv325	96.7	1.0 (4)	2.0 (3)	2
				1.7	>3.5	
B						
HSC4	III	Kv325	89.9	1.0 (12)	4.0 (12)	5
HSC5	III	L25	96.1	1.0 (6)	>2.0 (2)	2
HSC6	III	Kv325	93.5	0.2 (6)	2.0 (6)	4
HSC7	III	Kv325	90.6	2.7 (11)	>7.0 (7)	1
HSC8	I	Vk02/012	90.8	1.8 (11)	5.0 (6)	4
HSC11	III	Kv325	91.1	2.0 (9)	6.0 (7)	5
HSC13	III	Kv325	92.6	1.3 (9)	6.0 (7)	2
HSC15	I	Vk02/012	100.0	0.0 (0)	0.0 (0)	2
HSC21	III	Kv325	98.0	>1.0 (1)	2.0 (3)	4
HSC23	III	Kv328h5	96.0	1.0 (6)	1.0 (2)	5
HSC28	III	Kv325	97.3	0.0 (1)	>4.0 (4)	1
HSC33	III	Kv325	96.9	1.0 (4)	1.0 (2)	1
HSC34	III	Kv328h5	96.0	3.0 (4)	>1.0 (1)	4
HSC35	III	Kv325	96.5	0.3 (4)	2.0 (3)	4
HSC36	III	3A7	86.2	2.0 (18)	3.0 (8)	1
HSC37	I	A30	95.2	2.5 (7)	>4.0 (4)	2
HSC38	III	Kv325	96.9	2.0 (3)	4.0 (5)	1
HSC43	I	L11	92.4	1.6 (13)	>2.0 (2)	2
				>1.4	>3.1	

[a] A. Clones isolated during initial selection. B. Clones isolated from the LNA3HLn shuffled library. Only CDR1 and 2 were included in the calculations of the CDR R/S ratio.

(74%) of Abs using the light chain germ-line genes kv325 and Vk02/012. By searching the database we previously found that these two germ lines are frequently used by human hybridoma Abs directed at both autoantigens and non-self Ags (35, 36) Further, many Ag-specific Fabs selected from different combinatorial libraries have also revealed a frequent usage of these two light chains in Abs, although at a somewhat lower level than that reported here. The frequent use of the vk02/012 and kv325 light chains generally has been ascribed to the "plastic" qualities of these light chains such that they can pair with dominant heavy chains with retention of Ag binding (37, 38). The somewhat more frequent use of these light chains in polyreactive Fabs compared with specific Fabs may reflect the desirability of plastic qualities in a polyreactive Ab. Interestingly, Ichiyoshi and Casali, in their study on chimeric monoclonal and polyreactive Abs, also associated the kv325 light chain with the polyreactive phenotype (2).

Having access to a large number of sequences from human polyreactive and specific Abs, we examined the sequences for evidence of features beyond those discussed above that could be associated with polyreactivity. However, no such features were readily apparent. Extensive somatic mutation was evident in both heavy and light chains, but no unequivocally characteristic patterns emerge. In view of the established importance of HCDR3 discussed below, this region was analyzed in greater detail. The HCDR3 length and the amino acid usage were similar for the polyreactive and the Ag-specific Fabs. With respect to the distribution of amino acids, most of the HCDR3 of the polyreactive Fabs contained clusters of glycines, serines, or prolines, which might permit multiple alternative loop conformations. However, such clusters were also observed in the Ag-specific Fabs. Interestingly, Casali and co-workers (3) reported an HCDR3 motif of five amino acids, RFLEW, which they observed in two distinct different IgG polyreactive Abs

cloned from different individuals. This same motif is also found in the HCDR3 of one of our human polyreactive Fab fragments, LEG30 (Fig. 1), and therefore may well contribute to polyreactivity. Generally, however, we were not able to correlate polyreactive behavior with amino acid sequence in HCDR3 or elsewhere.

In additional studies, we conducted a series of manipulations on a prototype polyreactive human Fab (LNA3), chosen because of its marked polyreactivity with moderate affinity for a range of Ags. To study the contribution of heavy and light chains to Ag binding, we panned two shuffled libraries, LNA3HLn and LNA3LHn, against a panel of three autoantigens to yield a panel of polyreactive Fabs. Construction of shuffled libraries is complementary to heavy-light chain interchange experiments (39, 40), with the advantage that a large repertoire of heavy-light recombinations can be rapidly evaluated. By analyzing the sequences of polyreactive Fabs we found that a limited set of heavy chains was involved. Of the polyreactive Fabs retrieved from the heavy chain-shuffled library, many of the heavy chains were identical with those retrieved from the initial selection procedure and included the original parent Fab LNA3 heavy chain. In contrast, the polyreactive Fabs selected from the light chain-shuffled library contained a new set of unique light chains. These results support the importance of the heavy chain for Ag binding of the Fab LNA3. This result is in accordance with earlier studies, including those of Radic et al. (39). The recurrent selection of certain heavy chains in this experiment together with the likelihood that some of the chains in Figures 1 and 2 are clonally related suggest that the polyreactive Ab repertoires present in the libraries studied may be relatively limited.

Next, we specifically concentrated on the role of the HCDR3 region in the polyreactive behavior of the prototype Fab. The importance of HCDR3 for polyreactivity has been suggested by a number of studies (2, 41–43). In the most recent studies, Ichiyoshi and Casali generated a panel of chimeric Abs by combining different regions of a polyreactive and a monoreactive Ab and evaluating the binding pattern of the chimeric Abs (2). They found that the polyreactive Ab lost its reactivity pattern when the HCDR3 was exchanged with the HCDR3 from the monoreactive Ab, whereas the Ab remained polyreactive following exchange of the FR1 to FR3 regions. They also showed that the monoreactive Ab, upon grafting of the HCDR3 from the polyreactive Ab, became polyreactive. Similar grafting experiments between a polyreactive and a monoreactive Ab were performed by Crouziner et al. (11), and similar conclusions were reached. In both of these studies, grafting was conducted into an Ab of the same V_H germ line as the parent. We chose to "pressure" the polyreactive HCDR3 even more by grafting it into a completely different framework using a different V_H family. The donor Ab was from the V_H4 family and the acceptor was from the V_H1 family. Also, the V_L germ line of the acceptor Ab was different from that of the original polyreactive Ab. The chimeric Fab retained considerable affinity for a panel of different auto- and exogenous Ags, demonstrating the importance of the HCDR3. The frameworks may, however, still have some influence on polyreactivity (11), since the V_H and V_L frameworks of the grafted Fab were found in other polyreactive Abs (see Tables I and II).

To further evaluate the importance of the HCDR3 loop for the polyreactive phenotype, a constrained peptide derived from the HCDR3 of Fab LNA3 was synthesized. This peptide, PEPLNA3, was able to mimic the binding pattern of the entire Fab LNA3 and further inhibit the binding of the parent Ab to a panel of Ags. In contrast, a constrained peptide derived from the HCDR3 of a specific Fab exhibited no binding to these Ags. This result clearly demonstrates the major importance of the HCDR3 for the polyreactive phenotype of this Ab.

One potential contributor to polyreactive behavior is conformational flexibility in the Ab-combining site. To explore this possibility, we examined the dependence of Ag binding on temperature for two polyreactive Abs. One of the Fabs that was polyreactive at 37°C demonstrated close to monoreactive binding at 4°C (Fig. 3, *a* and *g*). The effect of temperature on the other Fab was less marked, but, nevertheless, a trend toward less polyreactive behavior at the lower temperature could be discerned (Fig. 3, *h* and *i*). There is, therefore, a strong suggestion from these data that conformational flexibility, which the earlier data would associate with the HCDR3, is important in Ab polyreactivity.

In conclusion, a body of evidence in the literature has begun to implicate the HCDR3 region as being crucial for Ab polyreactivity. This work confirms these findings and goes further in demonstrating the ability of a peptide corresponding to the HCDR3 to mimic polyreactivity. The importance of flexibility in polyreactive behavior is emphasized by the sensitivity of the phenomenon to temperature. No other overwhelming structural or genetic correlates of polyreactivity were identified despite the large panel of Abs studied. There were some interesting trends, such as the common occurrence of certain light chains, the low occurrence of V_H3 heavy chains, and the high degree of somatic mutation. A more complete understanding of polyreactive Abs now demands the solution of the structures of such Abs in complex with a number of different Ags.

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